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Shape variation in the skull within and between wild populations of the raccoon dog (*Nyctereutes procyonoides*) in Japan

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Abstract. Patterns of evolution can be biased by patterns of variability (propensity to vary). However, the effects of variability on evolution at the geographic and inter-subspecies levels have not been investigated extensively. Variation between different populations is the result of evolution. Individual variation within a single population can be indicative of species variability. Therefore, comparing patterns of variation within and between populations can reveal the effects of variability on evolution. In this study, I used two-dimensional geometric morphometric methods to evaluate variation in the shape of raccoon dog skulls within and between wild populations in Japan. In three of the populations observed, the dominant individual variation was cranial tilting. Cranial tilting is also a major variation between Japanese populations that has no correlation with climatic factors. However, differences in another morphological trait involving the frontal bone, parietal bone, and zygomatic arch dominated the variation between the Hokkaido population and other populations in Japan that are often considered to be a different subspecies. This morphological trait was correlated to snowfall. In conclusion, at the intra-subspecies level, morphological evolution in the skull is neutrally driven by variability. However, at the inter-subspecies level, adaptation may have a relatively larger effect on morphological evolution.

Key words: airorhynchy, cranial tilting, geographic variation, klinorhynchy, variability.

Darwin (1859) wrote that individual variation of traits tend to be high when inter-specific variation of those traits within a genus is also high. This observation can be applied to the concepts of variability and evolvability, in that high variability and evolvability of traits promotes the evolution of those traits within particular genera. Recently, evolutionary biologists have recognized the importance of evolvability (Futuyma 2010). Evolvability is the ability of populations to respond to selection (Houle 1992) and depends on phenotypic variability, i.e., propensity to vary (Wagner and Altenberg 1996; Kirschner and Gerhart 1998). Some researchers have referred to traits with relatively wide variation as the "line of least resistance" to evolution (Schluter 1996; Klingenberg 2010). Individual variation within populations, as a reflection of variability, is the raw material for evolution. Within-population variation therefore biases, guides, or constrains micro-evolution (Barton et al. 2007; Hendrikse et al. 2007; Wilson 2013). In this context, variability and evolvability can be roughly evaluated by examining individual variation within a single population (Wagner and Altenberg 1996; Klingenberg 2005, 2008; Pigliucci 2008). On the other hand, variation between populations results from evolution, including local selection and neutral evolution. Therefore, comparisons of the patterns of variation within populations to those between populations are comparisons between variability or evolvability and evolution.

Several previous studies have suggested that lines of least resistance (or patterns of variability and evolvability) are similar to evolutionary changes (Kluge and Kerfoot 1973; Sokal 1976; Begin and Roff 2004; Marroig and Cheverud 2005; Renaud et al. 2006, 2011; Young and Badyaev 2006; Hunt 2007), although Rohlf (1983) claimed that several historical studies (Kluge and Kerfoot 1973; Sokal 1976) were flawed by statistical artifacts. Other researchers have reported several cases of deviation from lines of least resistance during evolution, but agreed that the pattern of evolution is influenced by lines of least resistance (Merilä and Björklund 1999; Badyaev and Hill 2000; Ackermann and Cheverud 2004). However, whether the degree of influence variability on the

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pattern of evolution differs at different levels of evolution has not been tested. That is, the effect of variability may differ at the inter-subspecies level from that at the intra-subspecies level of evolution. In addition, in previous studies, morphological traits have often been translated into numerical scales that make it difficult to discuss the actual shape of morphological characters.

Cranial tilting between snout and neurocranium (deformation from klinorhynchy to airorhynchy) is the most significant skull shape variation within a single population of raccoon dogs *Nyctereutes procyonoides* in Gifu Prefecture, Japan (Asahara 2013). This shape variation was not associated with sex or size and was, therefore, a purely individual variation in shape. The degree of cranial tilting is a highly variable and evolvable trait in this population (Asahara 2013). However, it is still unclear whether this type of variation also dominates the variation within or between other raccoon dog populations. In this study, I examined several populations in Japan, including two subspecies, *N. p. viverrinus* distributed in Honshu, Kyushu, Shikoku and the other peripheral islands south of the Tsugaru Strait, and *N. p. albus* distributed in Hokkaido and its peripheral islands (Saeki 2009). The objective of this study was to examine the degree to which variability affects the evolution of skull shape at the intra-subspecies and inter-subspecies level, and to describe the geographic variation in skull shape in Japanese raccoon dogs. In this study, I therefore compared the variation in skull shape within and between populations, and investigated whether either intra-subspecies or inter-subspecies level variation is similar to cranial tilting, i.e., a line of least resistance.

Materials and methods

I examined 177 raccoon dog specimens (*Nyctereutes procyonoides*) from 8 localities in Japan (Hokkaido, 63 specimens; Fukushima, 2; Niigata, 8; Kanto, 6; Shizuoka, 3; Gifu, 68; Shimane, 22; and Miyazaki, 5; Fig. 1; Appendix 1). Among specimens examined, 52 specimens were male and 44 specimens were female. All specimens were considered to be mature as they exhibited complete adult dentition and closed basisphenoid-basioccipital sutures.

I used a two-dimensional geometric morphometric technique (Zelditch et al. 2004) to evaluate variations in skull shape. Fifteen landmarks on lateral-view photographs of the skulls (Fig. 2) were digitized using tpsDig2 (Rohlf 2010). After generalized Procrustes superimposition, coordinate data of the landmarks were separated

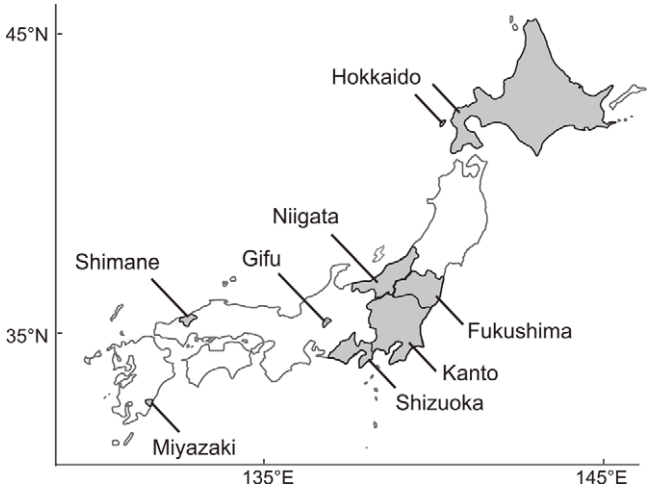

Fig. 1. Locations of the 8 raccoon dog (*Nyctereutes procyonoides*) populations studied.

into size (CS: centroid sizes, square root of the sum of squared distances from each landmark to the centroid of the landmarks) and multivariate shape (partial warp scores) by using tpsRelw (Rohlf 2010). Shape variations were extracted using relative warp analysis, which is a principal component (PC) analysis of partial warp scores, using tpsRelw (Rohlf 2010) or Minitab 14 (Minitab, Inc., PA, USA). The resulting PC axes were considered to be major axes of shape variations. PC analyses were performed four times, using total, Hokkaido, Gifu, or Shimane datasets to calculate major lines of variation (PC axes) for the total dataset (total PC axes) and within local populations (with > 20 individuals; local PC axes). To compare these PC axes, i.e., intra-population and inter-population variation, I calculated inner products of total and local PC vectors. The inner products of total and local PC vectors measure the similarity of shape deformations along PC axes. The inner products approach 1 or –1, when the axes of the shape deformations under consideration are similar. When the inner products approach 0, the axes of the shape deformations under consideration are different. To visualize intra-population variation, generalized Procrustes superimposition and relative warp analyses were also computed for each local population with > 20 individuals (Hokkaido, Gifu and Shimane populations). The PCs that resulted from these analyses are denoted as local^2 PCs. Shape deformations along major PC axes were illustrated using tpsSpline (Fig. 2; Rohlf 2004). Sexual dimorphism, size factors, and shape differences between populations were tested using a general linear model (GLM) of the major PC scores. Prior to GLM statistics, population data were

evaluated using the Anderson-Darling test for normality and Levene's test for equality of variances. Tukey's test was used for pairwise comparisons. To clarify relationships between skull shape and climates, I performed Spearman's rank correlation analysis of the local means of major PC scores on climatic factors at each locality (coldest-month mean temperature, warmest-month mean temperature, precipitation, and snowfall). Climatic data was obtained from the Japan Meteorological Agency website (data from 1981–2010). The monitoring points used were "Sapporo" (in Sapporo City) for Hokkaido population, "Fukushima" (in Fukushima City) for Fukushima population, "Niigata" (in Niigata City) for Niigata population, "Tokyo" (in Tokyo City) for Kanto population, "Shizuoka" (in Shizuoka City) for Shizuoka population, "Kanayama" (in Gero City) for Gifu population, "Hamada" (in Hamada City) for Shimane population, and "Miyazaki" (in Miyazaki City) for Miyazaki population. Statistical analyses were performed using Minitab 14 (Minitab, Inc., PA, USA).

Results

In the present study, the following pairs of axes had similar shape deformations; total PC1 and Hokkaido PC1, total PC2 and Gifu PC1, total PC2 and Shimane PC1, and total PC2 and Hokkaido PC1 (Table 1). Local^2 PC scores were totally correlated to local PC scores at each location (correlation coefficients were larger than 0.999). Shape deformations along the major PC axes are shown in (Fig. 2). Total PC1 explained 21.9% of the total shape variation and total PC2 explained 15.6% of the total variation. Shape deformations along the total PC1 axis included the degree of development (relative size) of the frontal bone, parietal bone, or top of the neurocranium, and the angle of the zygomatic arch. As total PC1 score increased, the suture between the frontal and parietal bone moved dorsally and the anterior part of the zygomatic arch moved to ventrally (Fig. 2). Total PC2 indicated cranial tilting or dorsoventral flexion between the snout and neurocranium, or, in other words, shape deformation of the skull from klinorhynchy to airorhynchy. As total PC2 score increased, the anterior part of the snout moved dorsally, the posterior part of the snout moved ventrally, the anterior part of the neurocranium moved ventrally, and the posterior part of the neurocranium moved dorsally (Fig. 2).

Within the Gifu population, deformation along the Gifu^2 PC1 axis showed cranial tilting similar to that of total PC2 (Fig. 2). As Gifu^2 PC1 score increased, the snout and neurocranium moved as described for total PC2. The Gifu^2 PC2 axis showed differences in parietal bone and anterior part of the zygomatic arch, similar to the results reported by Asahara (2013).

Within the Shimane population, deformation along the Shimane^2 PC1 axis showed cranial tilting similar to that of total PC2 (Fig. 2). As Shimane^2 PC1 score increased, the snout and neurocranium moved as described for total PC2. As Shimane^2 PC2 score increased, the anterior parts of the snout moved toward the posterior, the posterior parts of the snout moved toward the anterior, the top of the neurocranium moved dorsally and anteriorly, and the bottom of the neurocranium moved ventrally and posteriorly (Fig. 2). As a result, the snout appeared to decrease in size relative to the neurocranium as Shimane^2 PC2 score increased (Fig. 2).

Within the Hokkaido population, both Hokkaido^2 PC1 and Hokkaido^2 PC2 reflected cranial tilting and the relative size of the snout to that of the neurocranium. Dorso-ventral movement of the anterior and posterior snout and the anterior and posterior neurocranium (i.e., the anterior part of the snout and posterior part of the neurocranium moved ventrally and the posterior part of the snout and anterior part of the neurocranium moved dorsally) were included in Hokkaido^2 PC1, while the antero-posterior movement of the anterior part of the snout and the top and bottom of the neurocranium (i.e., the anterior part of the snout and top of the neurocranium moved posteriorly and bottom of the neurocranium moved anteriorly) were included in Hokkaido^2 PC2. In fact, when Hokkaido^2 PC1 was adjusted by a factor of 0.06 and Hokkaido^2 PC2 was adjusted by a factor of –0.04, the resulting composite image displayed cranial tilting that appeared similar to that of total PC2, Gifu^2 PC1 and Shimane^2 PC1 (Fig. 2).

Within each locality, variation of total PC1 and total PC2 scores were normal and equally distributed (Anderson-Darling test, $P > 0.05$; Levene's test, $P > 0.05$). Total variation in both total PC1 and total PC2 was associated with locality ($P < 0.05$), but not sex or skull size ($P > 0.05$). Bivariate plots of total PC1 against total PC2 implied shape differences between populations (Fig. 3). Between-population pairwise comparisons of total PC1 and total PC2 scores are shown in Tables 2 and 3, respectively. The total PC1 score for the Hokkaido population differed from those of the other Japanese populations, i.e., *N. p. viverrinus* but there were no significant differences among the total PC1 scores of the other Japanese popula-

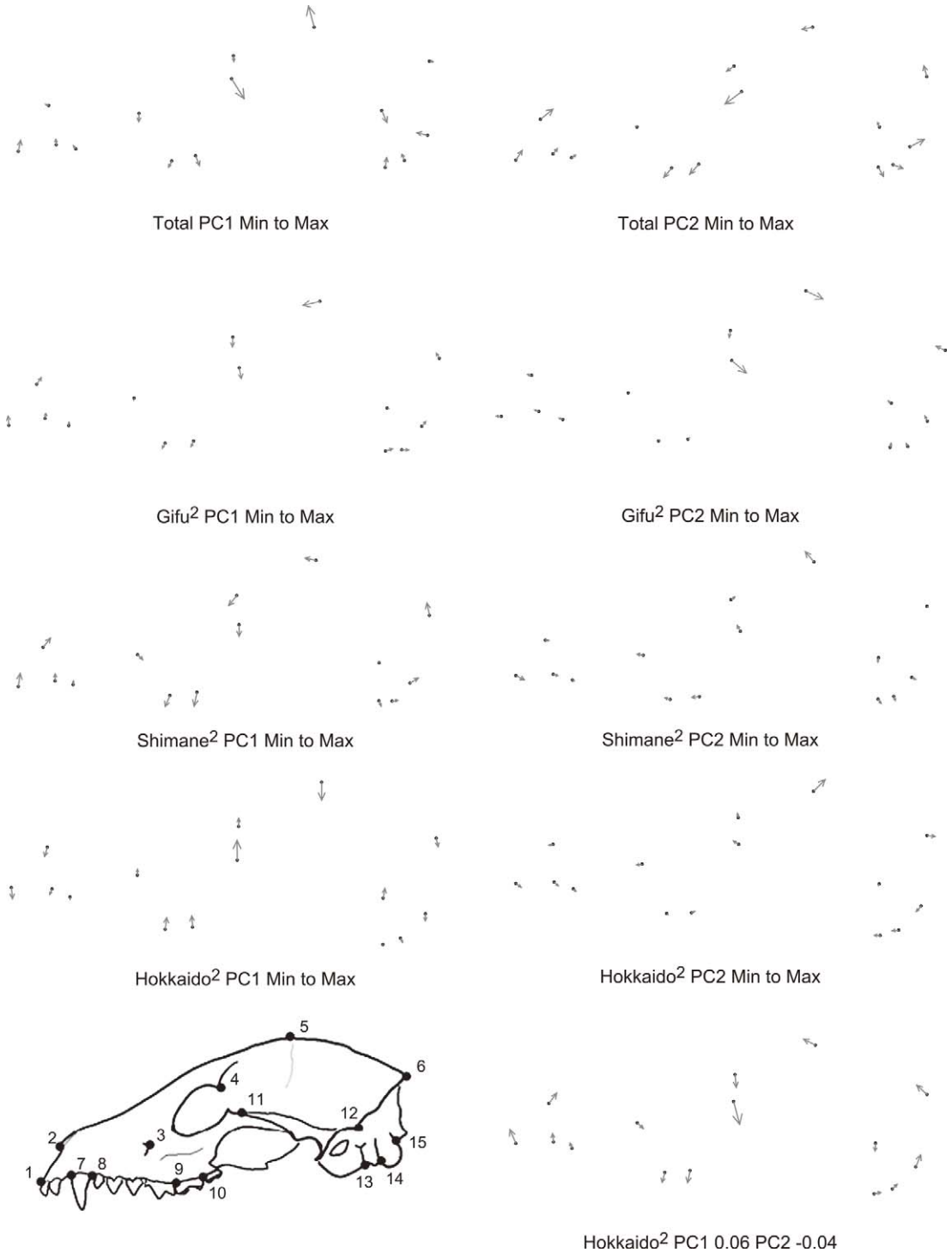

Fig. 2. Shape deformations along principal component (PC) axes and landmarks used to compare the skulls of raccoon dogs (*Nyctereutes procyonoides*; modified from Asahara, 2013). Shape deformations along PC axes are indicated by arrows. Shape deformations along the PC1 axis that were calculated from the total dataset (total PC1) reflect differential development of the frontal bone and differences in the angle of the zygomatic arch. Cranial tilting (klinorhynchy to airorhynchy deformation) is the most significant shape deformation in Gifu and Shimane populations (shown as Gifu^2 PC1 and Shimane^2 PC1). If Hokkaido^2 PC1 and PC2 are combined (Hokkaido^2 PC1 0.06, PC2 –0.04), cranial tilting becomes a significant shape deformation in Hokkaido population.

Table 1. Inner products of total and local principal-component-vectors (PC vactors)

	Total PC1	Total PC2	Total PC3	Total PC4
Hokkaido PC1	**–0.68**	**0.53**	–0.43	0.18
Hokkaido PC2	0.29	0.21	0.13	**0.83**
Hokkaido PC3	–0.07	**0.51**	**0.58**	–0.32
Hokkaido PC4	0.34	0.46	–0.12	–0.24
Gifu PC1	–0.50	**0.71**	0.24	–0.39
Gifu PC2	0.23	0.19	**0.64**	0.49
Gifu PC3	0.26	**0.60**	–0.40	0.35
Gifu PC4	0.01	–0.07	0.17	–0.22
Shimane PC1	–0.31	**0.89**	–0.03	–0.13
Shimane PC2	–0.13	–0.19	–0.50	**–0.52**
Shimane PC3	0.12	0.11	0.00	–0.13
Shimane PC4	–0.25	–0.20	**0.54**	–0.39

Boldface type indicates similarity of local PC vectors to the total PC vector, as indicated by inner products that were greater than 0.5 or less than –0.5.

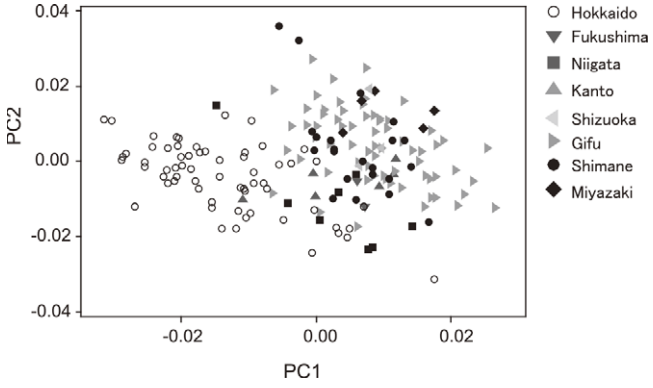

Fig. 3. Bivariate plots of total PC1 against total PC2.

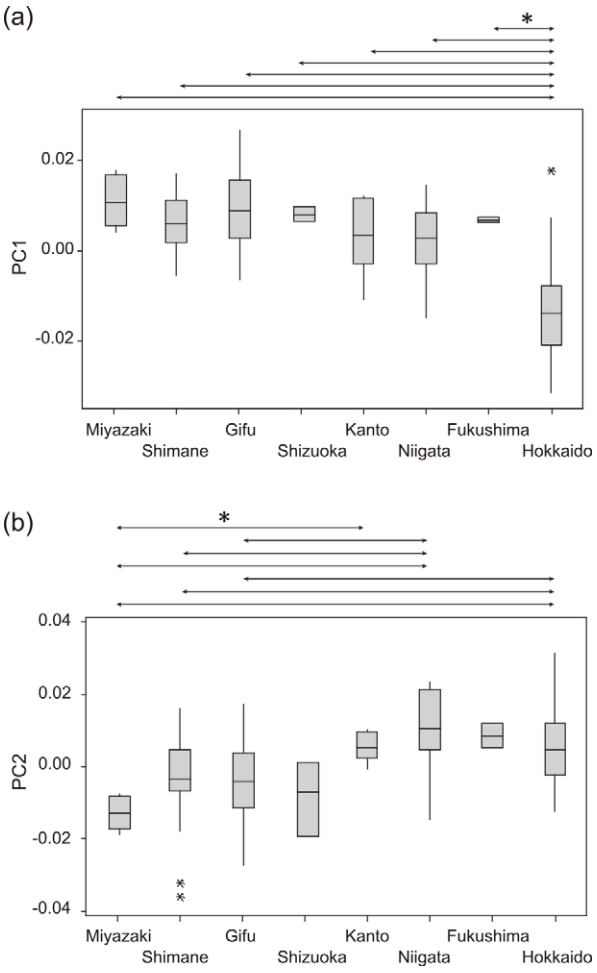

Fig. 4. Box plots of (a) the total PC1 scores and (b) total PC2 scores for each raccoon dog population. Boxes indicate quartiles, central-lateral bars indicate averages, vertical bars indicate the range of the specimens and asterisks indicate outliers. Significantly different pairs are indicated by arrow-lines.

Table 2. Results (*P*-value) of ANOVA-Tukey tests for differences between the total PC1 between localities

	Fukushima	Niigata	Kanto	Shizuoka	Gifu	Shimane	Miyazaki
Hokkaido	**0.026**	**0.000**	**0.000**	**0.001**	**0.000**	**0.000**	**0.000**
Fukushima		0.999	1.000	1.000	1.000	1.000	0.999
Niigata			1.000	0.983	0.525	0.982	0.733
Kanto				0.996	0.830	0.999	0.875
Shizuoka					1.000	1.000	1.000
Gifu						0.863	1.000
Shimane							0.957
Miyazaki							

Significant differences are indicated by boldface type.

tions, i.e., within *N. p. viverrinus* (Table 2; Fig. 4a). Total PC2 scores differed between several pairs of populations, including those within *N. p. viverrinus* (Table 3; Fig. 4b).

Total PC1 scores at each location were correlated to snowfalls (Table 4). As snowfall increased, total PC1 score decreased. However, total PC2 scores did not correlate to any of the climate factors evaluated (Table 4).

Table 3. Results (*P*-value) of ANOVA-Tukey tests for differences in the total PC2 scores between localities

	Fukushima	Niigata	Kanto	Shizuoka	Gifu	Shimane	Miyazaki
Hokkaido	0.999	0.636	1.000	0.498	**0.000**	**0.038**	**0.006**
Fukushima		1.000	1.000	0.651	0.631	0.721	0.165
Niigata			0.970	0.126	**0.002**	**0.013**	**0.001**
Kanto				0.610	0.322	0.519	**0.049**
Shizuoka					0.999	0.999	0.994
Gifu						1.000	0.539
Shimane							0.535
Miyazaki							

Significant regressions are indicated by boldface type.

Table 4. Spearman's rank correlations between major principal components (PCs) and local climates (*P*-value)

	PC1	PC2
Coldest-month mean temperature	0.208	0.208
Warmest-month mean temperature	0.529	0.659
Precipitation	0.801	0.950
Snowfall	**0.049**	0.136

Significance of regressions are indicated by boldface type.

Discussion

Local PCs and local^2 PCs were totally correlated, and therefore, shape deformations of local^2 PCs are considered to be virtually identical to those of local PCs. Cranial tilting was the most significant lateral shape variation within all of the populations observed (Fig. 2). The Gifu and Shimane populations are distant and independent, and the study areas were distinctly small. Therefore, my study was based on two independent investigations on two distinct populations and the result in cranial variability is reliable (Fig. 1). My results indicated that there were no sexual dimorphisms in total PC1 and PC2 and were based on mature specimens, which should not be affected by age. Therefore, cranial tilting is considered to be the most variable and evolvable trait of Japanese raccoon dogs and therefore constitutes the line of least resistance. Variation in cranial tilting has also been reported between different breeds of dog (Fondon and Garner 2004) and between local populations of wolf (Milenković et al. 2010). This cranial tilting occurs between the snout region and the neurocranium region, which constitute separate modules (Drake and Klingenberg 2010). In addition, variation in cranial tilting among dog breeds is related to polymorphisms at a single gene locus: a tandem repeat in a regulatory region of the *Runx2* gene (Fondon and Garner 2004). A mutation at this locus may affect relative growth of the dorsal midface (Fondon and Garner 2007). Cranial tilting in raccoon dogs may result from similar mechanism. Canid species have intraspecific polymorphisms of this tandem repeat length of the *Runx2* gene (Fondon and Garner 2004). This suggests that cranial tilting is highly variable and evolvable in canids, in general.

Cranial tilting, reflected in total PC2, was also observed as an inter-population variation in the present study (Fig. 2). The inner products of the following pairs of major PC vectors approached 1 or –1: total PC1 and Hokkaido PC1, total PC2 and Gifu PC1, total PC2 and Shimane PC1, and total PC2 and Hokkaido PC1 (Table 1). These were the only pairs of axes with inner products that were greater than 0.5 or less than –0.5 (Table 1). Therefore, the cranial tilting observed between populations was morphologically similar to that observed within the Hokkaido, Gifu, and Shimane populations.

Total PC1, the most significant variation in skull shape among populations of Japanese raccoon dogs, is reflected in the degree of frontal bone development and the angle of the zygomatic arch (Fig. 2). This type of variation was not an obvious within-population variation (Table 1), except in Hokkaido (Hokkaido^2 PC1). Total PC1 and Hokkaido PC1 were similar (Table 1). This similarity may result from the relatively large sample area in Hokkaido and may not indicate true within-population variation, that is, it is possible that inter-population variation affected the results. I was not able to evaluate separate localities in Hokkaido because detailed location information was not available for most specimens. The result of the present study may indicate that total PC1 differed from the pattern of variability, or the line of least resistance in Japanese raccoon dogs, especially in *N. p. viverrinus*.

Total PC1 scores for the Hokkaido population differed from those of the other Japanese populations, whereas those for the other Japanese populations did not differ from each other (Table 2; Fig 4a). Therefore, total PC1 reflects a difference in shape between the Hokkaido raccoon dog population, i.e., *N. p. albus* and the other Japanese populations, i.e., *N. p. viverrinus*. Shape deformations along total PC1 axis included differences in the top of the neurocranium and the anterior part of the zygomatic arch; Hokkaido raccoon dogs had relatively flat neurocrania and higher zygomatic arches in lateral view (Figs. 2 and 4a). Masticatory muscles, such as the temporal and masseter muscles, attach at these positions. Previous studies of skull measurements indicate that these subspecies have different skull morphology, and this difference in morphology may reflect differences in dietary habits or climate (Yoshiyuki 1988; Haba et al. 2008). My shape deformation results (Figs. 2 and 4a), and the correlations between total PC1 scores on climatic factors (Table 4), support these previous suggestions. On the other hand, the total PC2 scores for the Hokkaido population differed from those of Gifu, Shimane, and Miyazaki populations, but did not significantly differ from those of other populations (Table 3; Fig. 4b). In addition, total PC2 was not correlated to climatic factors (Table 4). My results indicate that eastern *N. p. viverrinus* tended to have more airorhynchous skulls (with higher total PC2 scores) than western *N. p. viverrinus*, which had relatively klinorhynchous skulls (with lower total PC2 scores) (Figs. 2 and 4b). However, total PC2 scores seem to randomly differ between populations because PC2 scores for distant populations (e.g., between Kanto and Miyazaki populations) tended to differ to a larger degree than those of close populations (e.g., Kanto and Gifu populations) (Fig. 4b). Therefore, I consider that the evolution of cranial tilting was nearly neutral and was guided by the variability, evolvability, and line of least resistance in the skull. According to previous molecular phylogeographic studies, Honshu and Shikoku raccoon dog populations could not be separated from one another and the variation of the Hokkaido population was nested within the variation of the Honshu and Shikoku populations (Kurose et al. 2010, 2012; Kim 2011; Kim et al. 2013). This low degree of genetic separation supports the idea that total PC1 reflects a history of rapid adaptation that was different for the Hokkaido population than for the other populations after the Hokkaido population was isolated by the Tsugaru Strait. On the other hand, total PC2 reflects neutral evolution that was led by variability, evolvability, and random drift.

According to the results of the present study, the evolution of skull shape in Japanese populations, except for Hokkaido population, was guided by variability, evolvability, or line of least resistance, while the evolution of skull shape in the Hokkaido population was greatly affected by adaptation. Variability and evolvability are the most important factors in the intra-subspecies evolution of Japanese raccoon dogs. In contrast, the differentiation between subspecies is affected to a greater degree of adaptation than by the degree of variability and evolvability. Further comparisons between different levels of variation in different taxa would provide a better understanding of how evolution is constrained or guided by the variability and evolvability of organisms.

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Appendix 1.

Specimens examined

Locality	Specimen number				*n*	Institute
Hokkaido	9905, 11429, 11430M, 11431F, 11432F, 11433, 11434, 11435, 11436, 11439, 11440, 11441, 11443, 11444, 11446, 11447, 11448, 11449, 11450, 11453, 11454, 11455, 11457, 11459, 11461, 11464, 11466, 11467, 11468, 11471M, 11472, 11473, 11474, 11475, 11477, 11478, 11479, 11480, 11481, 11482, 11483, 11485, 11497, 24940, 46029, 47615, 47616, 47619, 47623, 47624, 47625				51	Museum of Botanic Garden, Hokkaido University
	27278M, 27278M, 27280M, 27280M, 27324M, 27325F, 27325F, 27329M, 27362M, 27362M, 27363F, 27363F				12	National Museum of Nature and Science
Fukushima	9537, 25774				2	National Museum of Nature and Science
Niigata	1874F, 12091, 12190F, 12191M, 14650M, 15816M, 17628, 31488M				8	National Museum of Nature and Science
Kanto	21761, 23542, 23543, 25716, 25776, 34439F				6	National Museum of Nature and Science
Shizuoka	28702M, 28703M, 28708F				3	National Museum of Nature and Science
Gifu	1, 7F, 27, 32, 34, 35, 36M, 246M, 247M, 251M, 255F, 256F, 257M, 258F, 259M, 260M, 261F, 262F, 264M, 267M, 270M, 273F, 274M, 278M, 286F, 287F, 288F, 289F, 291F, 293M, 294M, 295M, 296M, 299M, 301F, 303F, 307M, 312M, 313F, 314F, 315M, 319F, 321M, 322F, 323F, 328F, 333F, 336M, 337F, 342F, 343F, 344F, 345F, 346M, 349M, 352M, 357M, 361F, 362M, 364M, 367M, 368M, 369F, 371M, 374M, 377M, 378M, 380F				68	Primate Research Institute, Kyoto University; data were from Asahara (2013)
Shimane	75-1, 75-2M, 75-5, 75-6M, 75-7, 75-8, 75-13, 75-14, 75-16F, 75-17F, 75-18, 75-22, 75-23, 75-27, 75-28, 75-30, 75-31, 75-32, 75-33, 75-34, 75-35, 75-36				22	The Kyoto University Museum, Kyoto University *Specimen numbers are private number of T. Ito who donated specimens to the museum
Miyazaki	25738M, 27351M, 28176F, 28177F, 28185F				5	National Museum of Nature and Science

M: male; F: female